Reversal of Allergic Airway Hyperreactivity after Long-term Allergen Challenge Depends on γδ T Cells

Zhi-Hua Cui, Anthony Joetham, M. Kemal Aydintug, Yoon-Soo Hahn, Willi K. Born, and Erwin W. Gelfand

Program in Cell Biology, Department of Pediatrics, and Department of Immunology, National Jewish Medical and Research Center, Denver, Colorado

Long-term allergen exposure can attenuate inflammation and revert airway hyperreactivity to normal responsiveness. A model of such reversal was established in which airway hyperreactivity and inflammation in ovalbumin-sensitized and challenged mice were decreased after multiple daily airway challenges. This change in responsiveness and inflammation was associated with a transition from a helper T cell Type 2 to a helper T cell Type 1 cytokine-biased profile in bronchoalveolar lavage fluid. Cell transfer from long-term exposed mice into hyperreactive mice also restored normal airway responsiveness, establishing the mechanism underlying the reversal of the hyperreactivity as active suppression, but did not affect eosinophilic airway inflammation. Conversely, airway hyperreactivity, suppressed as a result of long-term allergen exposure, could be reestablished by depleting γδ T cells, in particular Vγ4$^+$ cells. Antigen-specific tolerance of αβ T cells or suppression by non-γδ T cells did not play a role in the reversal to normal airway responsiveness and γδ T cells did not play a role in the regulation of the allergic inflammatory response. These findings show that normal responsiveness in previously hyperreactive mice, achieved after long-term allergen challenge, is based on several, apparently independent regulatory mechanisms. One of these, focused on airway responsiveness, involves active suppression and requires γδ T cells.

Keywords: cellular activation; cytokines; mast cells/basophils; signal transduction

Eosinophilic airway inflammation, helper T cell Type 2 (Th2) cytokine production, and airway hyperresponsiveness (AHR) are characteristics of the response of mice to allergen sensitization and challenge (1–4). These responses are typically induced by acute or short-term allergen challenge of sensitized hosts. Airway responses and underlying regulatory mechanisms after chronic or long-term allergen challenge, which may be more applicable to the clinical situation, are less well defined (5, 6). In animal models, chronic allergen challenge induces suppression of the Th2 response to allergen and reduces airway responsiveness and airway inflammation (7, 8). Reductions of late-phase asthmatic responses to allergen after long-term allergen challenge have also been reported in a clinical study (9).

The importance of T lymphocytes in the orchestration of immune responses and modulation of airway responsiveness and airway inflammation has been delineated. Whereas αβ T cells mediate airway inflammation and hyperresponsiveness (10, 11), γδ T cells have been found to prevent the development of AHR after allergen challenge in sensitized animals (12–14). The cytokines interleukin (IL)-4, IL-5, and IL-13, released from activated Th2 cells, are linked to increases in AHR, airway inflammation, and mucus hyperproduction (15, 16). Other cytokines such as IL-12 and IFN-γ released from Type 1 helper T (Th1) cells are thought to inhibit AHR and airway inflammation induced by allergen challenge (17–21).

On the basis of the findings attributing a regulatory role to γδ T cells in AHR, we hypothesized that γδ T cells are also involved in the mechanisms that abrogate or reverse airway hyperreactivity and inflammation after long-term allergen challenge. In the present study, we established a model of repeated allergen exposure that results in normalization of airway responsiveness in previously hyperreactive, allergic mice. Airway inflammation as well as levels of IL-4 and IL-5 in the bronchoalveolar lavage (BAL) fluid are decreased, whereas levels of IFN-γ, IL-12, and IL-10 are increased. Lung lymphocytes transferred from such mice into short-term sensitized and challenged hyperreactive mice suppressed airway hyperreactivity but not airway inflammation. Pulmonary γδ T cells in such mice increased as airway responsiveness normalized, suggestive of a counterregulatory response. Indeed, depletion of γδ T cells and, in particular, one Vγ-defined subset, abolished the suppressive effect of long-term allergen exposure as indicated by the reappearance of AHR, but without an increase in airway inflammation.

METHODS

Animals

Female BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, ME), and cared for at the National Jewish Medical and Research Center (Denver, CO). All experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee.

Sensitization and Airway Challenge

Mice were sensitized to chicken ovalbumin (OVA) (Grade V; Sigma, St. Louis, MO) by intraperitoneal injection of 20 μg of OVA emulsified in 2.25 mg of aluminum (InjectAlum; Pierce Biotechnology, Rockford, IL) in a total volume of 100 μl on Days 1 and 14. After sensitization, animals were challenged with nebulized OVA (1% in phosphate-buffered saline [PBS]) for 20 minutes either daily on Days 28, 29, and 30 (short-term challenge) or daily from Day 28 to Day 52 (long-term challenge). Airway responsiveness to aerosolized methacholine (MCh) was evaluated on Day 32 (short-term challenge) or Day 54 (long-term challenge). Control groups were either sensitized with OVA (IP) but challenged with PBS or were nonsensitized (Nv) but challenged with OVA (N). Experiments were designed so that the ages of all groups were the same at the time of analysis (Figure 1).

Assessment of Airway Responsiveness

Airway responsiveness to aerosolized MCh was measured on the basis of previously described procedures (2, 22). Briefly, anesthetized (pentobarbital, 75 mg/kg, administered intraperitoneally) mice were tracheostomized and mechanically ventilated with a volume-constant ventilator. Aerosols of PBS or MCh (1.5, 3, 6, and 12 mg/ml) were generated with an ultrasonic nebulizer, using a second ventilator. Lung resistance (R_L) and dynamic compliance (C_{Ldyn}) after each MCh provocation were instantly computed by the plethysmograph on the basis of changes in

(*Received in original form May 9, 2003; accepted in final form September 5, 2003*)

Supported by NIH grants HL-36577, HL-61005, and HL-65410, and by Environmental Protection Agency grant R825702.

Correspondence and requests for reprints should be addressed to Erwin W. Gelfand, M.D., National Jewish Medical and Research Center, 1400 Jackson Street, Denver, CO 80206. E-mail: gelfande@njc.org

Am J Respir Crit Care Med Vol 168. pp 1324–1332, 2003
Originally Published in Press as DOI: 10.1164/rccm.200305-634OC.R1 on September 11, 2003
Internet address: www.atsjournals.org

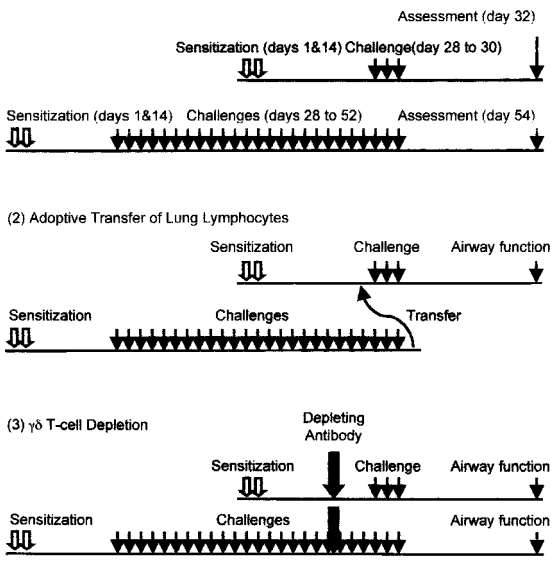

Figure 1. Experimental protocols. (*1*) To compare airway responses after short-term versus long-term allergen challenge, mice were sensitized to ovalbumin (OVA) by intraperitoneal injection on Days 1 and 14. Mice were challenged with aerosolized 1% OVA on Days 28, 29, and 30 (short-term challenge) or from Day 28 to Day 52 (long-term challenge). Airway responsiveness to aerosolized methacholine (MCh) was evaluated on Day 32 (short-term challenge) or Day 54 (long-term challenge). Control groups were either sensitized with OVA but challenged with phosphate-buffered saline or nonsensitized and challenged with OVA. (*2*) To evaluate the modulation of airway responsiveness by lung lymphocytes, T lymphocytes isolated from the lungs of long-term challenged mice were transferred to sensitized mice (1×10^8 cells per mouse) by injection through the tail vein. Recipients were then challenged with OVA three times and airway responsiveness was measured 48 hours later. (*3*) To define the regulatory effects of γδ T cells on airway responsiveness, hamster monoclonal antibodies against T cell receptor δ (GL3 and 403-A.10, 2.11, and UC-3) were injected (200 μg/mouse) via the tail vein into mice 7 days before measurement of airway responsiveness.

airflow, volume, and pressure. Dose–response curves for each concentration of MCh were constructed, using the maximum value of R_L and minimum value of C_{Ldyn}, after each provocative dose of MCh.

Inflammatory Cells, IgE, and Cytokine Levels in BAL Fluid

Immediately after assessment of airway responsiveness to MCh provocation, lungs were lavaged through the tracheal cannula with 1 ml of Hanks' balanced salt solution at room temperature. Total cell numbers were counted (Coulter Counter; Beckman Coulter, Fullerton, CA). Cytospin slides (Thermo Shandon, Pittsburgh, PA) were stained with LeukoStat (Fisher Diagnostics, Pittsburgh, PA) and differential cell counts were performed on the basis of at least 300 cells, according to standard morphologic criteria.

The levels of IL-4, IL-5, IL-10, IL-12, and IFN-γ in BAL fluid (BALF) and cell culture medium were measured in enzyme-linked immunosorbent assays (ELISAs). The assays were performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Cytokine levels in BALF were calculated by comparison with known cytokine standards with a detection limit of 4 pg/ml for each cytokine.

OVA-specific IgE levels were quantitated by ELISA as previously described, using standards established in the laboratory (23).

Histologic Examination of Lung Tissue

Lungs were fixed by injection of 1 ml of 10% formaldehyde through the tracheal cannula. The trachea was sutured to keep the fixative inside the lungs and airways. The paraffin-embedded lungs were sectioned (5 μm) and stained with hematoxylin and eosin to evaluate the general level of inflammatory cell infiltration, with periodic acid–Schiff to evaluate mucus production, and with immunohistochemical staining to identify eosinophils containing major basic protein in the lung tissue. The anti-major basic protein antibody was kindly provided by J. J. Lee (Mayo Clinic, Scottsdale, AZ). Histology analysis was done in a blinded manner by light microscopy linked to an image analysis system. Hyperplasia of goblet cells was expressed as the number of goblet cells per 100 μm of basement membrane.

Preparation of Lung Lymphocytes and Adoptive Transfer

Lymphocytes isolated from lung tissue were used for evaluation of lymphocyte composition in the lungs, for *in vitro* cell culture, and for adoptive lymphocyte transfer experiments. Lung digests were obtained as previously described (23). Briefly, lungs were removed and cut into small pieces (0.5 mm in diameter) and digested in 5 ml of digestion buffer (collagenase [0.5 mg/ml], 10% fetal calf serum, penicillin [100 U/ml], and streptomycin [100 mg/ml]) at 37°C for 60 min. The digested lung tissue was sheared with a 20-gauge needle and filtered through 45- and 15-μm pore size filters. Erythrocytes in the cell suspension were lysed with Gey's solution (ammonium chloride). Lymphocytes were obtained by gradient centrifugation of the cell suspension (Cappel LSMF; MP Biomedicals, Irvine, CA) and then filtering through a nylon–wool column. Lymphocyte composition (cell staining for CD3, CD4, CD8, and T cell receptor [TCR]-δ) was assessed by flow cytometry.

To monitor cytokine production of lymphocytes *in vitro*, isolated lung lymphocytes (4×10^5/well) were cultured with 10 μg of OVA *in vitro* for 48 hours and the levels of IL-4 and IL-5 in culture medium were measured by ELISA. To test modulation of allergen-induced AHR and airway inflammation by lung lymphocytes, we transferred 10^7 T lymphocytes isolated from the lungs of long-term challenged mice to short-term challenged mice by injection through the tail vein (Figure 1).

Depletion of γδ T Lymphocytes with Antibodies against the TCR

To further define the modulation of AHR and airway inflammation by γδ T cells, we depleted γδ T cells by injection of hamster monoclonal antibodies (200 μg/mouse) through the tail vein, 7 days before MCh provocation. A 1:1 mixture of pan-specific anti–TCR-δ monoclonal antibodies GL-3 and 403-A.10 (100 μg each) was used for general γδ T cell depletion, and monoclonal antibodies UC-3 (anti-Vγ4) and 2.11 (anti-Vγ1) were used to deplete $V\gamma4^+$ and $V\gamma1^+$ T cells, as previously described (12, 24–26). Hamster IgG (Jackson Laboratory) (Ham IgG in Figures 8–11), at the same concentration as the antibodies, was used for sham depletion in control mice. Depletion was confirmed by determining the residual number of γδ T lymphocytes in lungs and spleen by flow cytometry analysis, as previously described (12, 24). Three days after the treatment, each of the antibodies typically reduced the targeted cells in the spleen and in the lung by more than 95%.

Statistical Analyses

Data are expressed as means ± SEM for each group. The Kruskal–Wallis test was used first to ascertain that significant variance existed among the groups studied. The Mann–Whitney U test was then used to test statistical significance of the differences between two groups. A p value of less than 0.05 was considered significant.

RESULTS

Airway Responsiveness Is Normalized after Long-term, Repetitive Allergen Challenge

We first compared airway responsiveness of sensitized mice after short- and long-term allergen challenge by measuring airway responsiveness, R_L and C_{Ldyn}, to inhaled MCh. Short-term allergen challenge significantly increased airway responsiveness in

sensitized mice compared with controls (Figure 2). However, sensitized mice that experienced long-term challenges no longer exhibited AHR; the levels of airway responsiveness were similar to those of controls (Figure 2). These data implied that airway responsiveness after short- and long-term allergen challenges was regulated in distinct ways. The complete loss of AHR potentially reflected exhaustion of responsiveness or the emergence of an active counterregulatory mechanism.

Airway Inflammation and Mucus Production Are Reduced after Long-term Allergen Challenge

Short-term allergen challenge induced a typical inflammatory response in the airways of sensitized mice, with extensive inflammatory infiltrates around the airways and blood vessels and eosinophil infiltration in lung tissue and BALF (Figures 3 and 4). By contrast, mice exposed to long-term allergen challenges had reduced inflammation around the airways (Figure 3C), and fewer eosinophils in lung tissue (eosinophils were reduced from 169 ± 8/mm basement membrane in short-term challenged mice to 76 ± 5/mm basement membrane in long-term challenged mice) and BALF, but more lymphocytes in BALF compared with short-term challenged mice (Figure 4).

Short-term challenge also induced goblet cell hyperplasia and mucus production in the airways of sensitized mice compared with controls (Figure 3). However, the number of goblet cells was significantly reduced from 9 ± 0.4 cells per 100 μm basement membrane in short-term challenged mice to 5 ± 0.5 per 100 μm basement membrane after long-term challenge (n = 12 in each group, $p < 0.05$). The size of the goblet cells was also smaller after long-term challenge (Figure 3C).

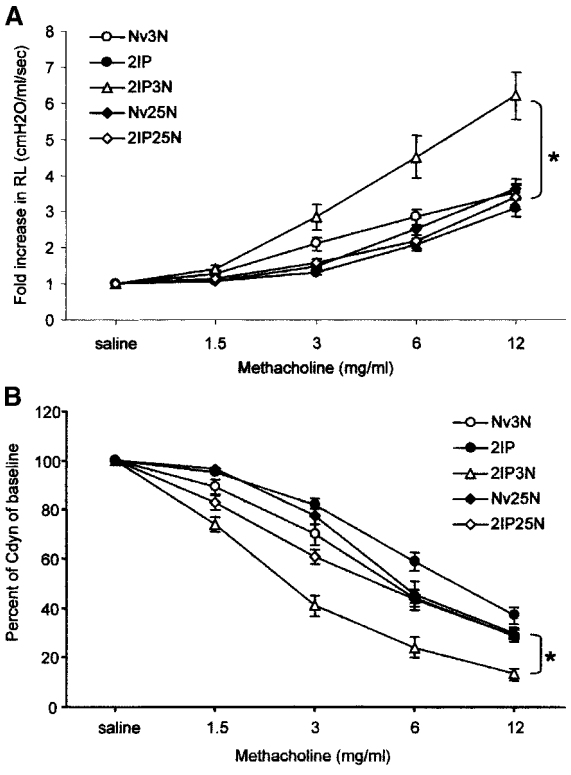

Figure 2. Airway hyperresponsiveness (AHR; short versus long). Increase in lung resistance (R_L; *A*) and decrease in dynamic compliance (C_{Ldyn}; *B*) after exposure to increased doses of MCh. Data represent means ± SEM (n = 8–14 mice in each group). Baseline R_L and C_{Ldyn} values were not altered by any of the treatments. *Significant difference ($p < 0.05$) between sensitized and short-term challenged mice (2IP3N, mice sensitized on Days 1 and 14, and challenged on Days 28, 29, and 30) and long-term challenged mice (2IP25N, mice sensitized on Days 1 and 14, and receiving daily challenges from Day 28 to Day 52). IP = sensitized; N = challenged; Nv = nonsensitized.

Cytokine Production and Allergen-specific IgE Are Altered (Th2 to Th1) after Long-term Allergen Exposure

Short-term challenge induced a Th2-type cytokine response in sensitized mice with elevated levels of IL-4 and IL-5 and decreased levels of IL-10 and IL-12 in the BALF compared with controls that were only sensitized but not challenged (2IP). In contrast, in long-term challenged mice, the levels of IL-10, IL-12, and IFN-γ were restored to levels seen in the sensitized but nonchallenged mice. The level of IL-5 in long-term challenged mice was decreased compared with that in short-term challenged mice, but it remained significantly higher than in unchallenged controls (Figure 5).

To confirm these findings at the cellular level, lung lymphocytes were isolated and cultured *in vitro* with OVA for 48 hours and the levels of IL-4 and IL-5 were measured by ELISA. The levels of IL-4 significantly increased from 147 ± 15 pg/ml in controls to 368 ± 55 pg/ml in short-term challenged mice and were decreased to 198 ± 29 pg/ml in long-term challenged mice (n = 8 in each group, $p < 0.05$). The levels of IL-5 increased from 137 ± 17 pg/ml in controls to 1018 ± 159 pg/ml in short-term challenged mice and were reduced to 403 ± 57 pg/ml in long-term challenged mice (n = 8 in each group, $p < 0.05$).

After sensitization and three challenges, OVA-specific IgE levels rose from undetectable to 298 ± 24 EU/ml. In long-term challenged mice, the levels of OVA-specific IgE were reduced to 102 ± 18 EU/ml. Levels of OVA-specific IgG1 and IgG2a showed little change.

Lung Lymphocytes from Long-term Allergen-exposed Donors Modulate Airway Responsiveness and Cytokine Production But Not Airway Inflammation in Hyperreactive Recipients

As shown in Figure 4, significantly increased numbers of lymphocytes in the BALF were detected after long-term challenge, suggesting their role in the modulation of airway responses. To investigate this possibility, 5×10^6 lymphocytes (more than 93% $CD3^+$, 59% $CD4^+$, 21% $CD8^+$, and 13% γδ) isolated from the lungs of long-term challenged mice were injected intravenously into sensitized mice before short-term challenge began. Airway responsiveness to MCh after short-term challenge was compared in the recipient mice. AHR elicited by short-term challenge was totally abolished after adoptive transfer of lung lymphocytes from the long-term challenged mice (Figure 6).

The levels of cytokines in the BALF of short-term challenged mice were also altered after transfer of lung lymphocytes from long-term challenged mice. The levels of IL-5 were decreased and the levels of IL-10, IL-12, and IFN-γ were increased after lymphocyte transfer (Figure 5).

However, adoptive transfer of lung lymphocytes did not affect the inflammatory cell composition of BALF in recipient, short-term challenged mice; that is, the predominant BALF eosinophilic inflammatory response was sustained in the recipient mice (Figure 7), and in the tissue (not shown). These data suggested that lung lymphocytes actively regulate airway responsiveness and cytokine production in the airways without altering eosinophilic airway inflammation.

γδ T Cell Numbers Are Increased in Lung after Long-term Allergen Challenge

We characterized the lymphocytes isolated from lungs (after gradient centrifugation) with monoclonal antibody against CD3

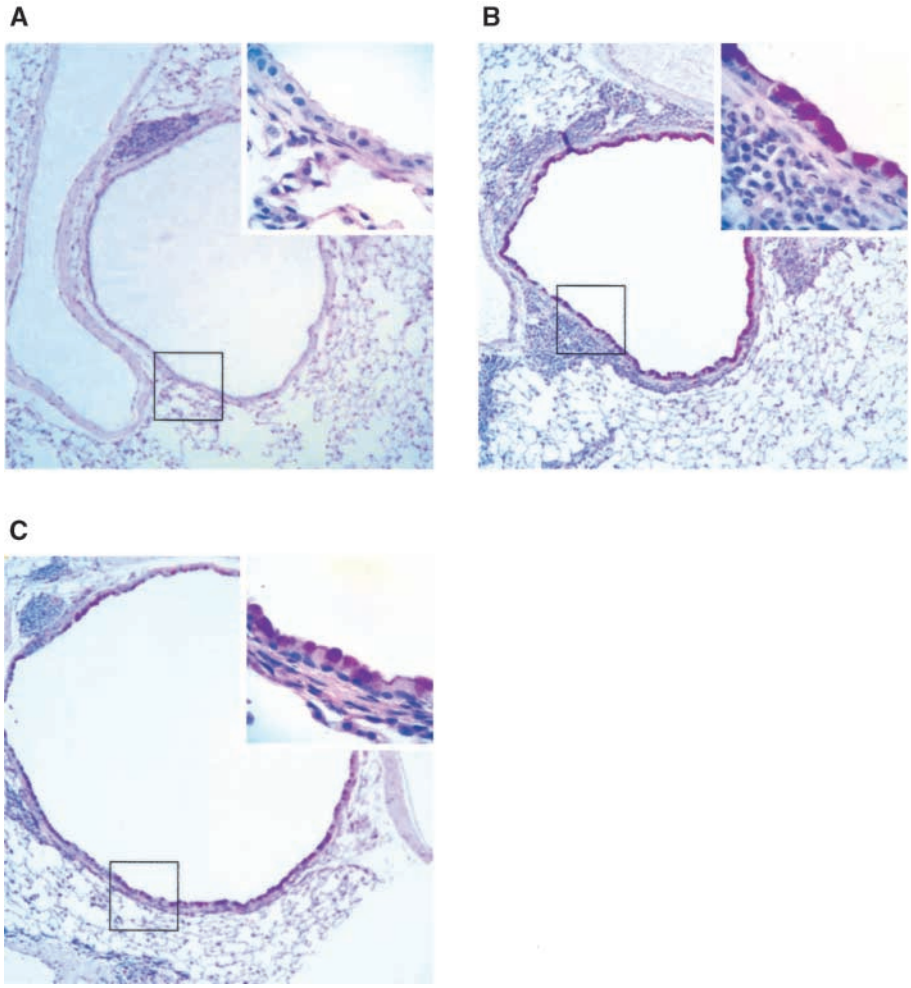

Figure 3. Inflammatory response and mucus production. Representative histology from controls (*A*), the short-term challenged group (*B*), and the long-term challenged group (*C*). Short-term allergen challenge induced an extensive inflammatory infiltration around airways and blood vessels and hypertrophy and hyperplasia of goblet cells compared with controls. Both inflammatory cell accumulation and mucus production were significantly attenuated after long-term allergen challenge (periodic acid–Schiff staining; original magnification: for general view, ×200; for insets, ×400).

and with antibody GL-3 against TCR-δ. The percentage of $CD3^+$ γδ T cells in the lungs was assessed by flow cytometry. Data showed that the percentage of $CD3^+$ γδ T cells in lungs increased from 3% in nonchallenged mice to 9% in short-term challenged mice and to 13% in long-term allergen-challenged mice ($p < 0.05$); $CD3^+CD8^+$ T cells increased (15, 17, and 21% in nonchallenged, short-term challenged, and long-term challenged mice, respectively) whereas $CD3^+CD4^+$ T cells decreased (68, 63, and 59% in nonchallenged, short-term challenged, and long-term challenged mice, respectively).

Depletion of γδ T Cells in Long-term Challenged Mice Restores AHR But Not Airway Inflammation

To evaluate whether γδ T cells play a role in the reversal of AHR after long-term challenge, we depleted γδ T cells, using monoclonal anti–TCR-δ antibodies (1:1 mixture of GL-3 and 403A-10). The mice were treated 7 days before airway responsiveness was assessed. Data obtained by flow cytometry 7 days after administration of the antibodies showed a 50 to 60% depletion of γδ T cells in both groups of mice by these antibodies, similar to our previous experience (12, 24). Airway responsiveness in short-term challenged mice tended to increase further after depletion of γδ T cells, as reported previously (12). AHR in long-term challenged mice was completely restored to levels as seen in short-term challenged mice after γδ T cell depletion (Figure 8). Hamster control immunoglobulin treatment showed no effect (not shown). To identify which subset of γδ T cells was the major regulator of airway function, we utilized hamster monoclonal antibody 2.11 and UC-3 to selectively deplete $V\gamma1^+$ and $V\gamma4^+$ γδ T cells, respectively. Flow cytometry showed a greater than 95% depletion of either subset of γδ T cells by the respective antibody, confirming previous results with these antibodies (27). After depletion of $V\gamma4^+$ γδ T cells, AHR was nearly fully reestablished in the long-term challenged mice; depletion of $V\gamma1^+$ γδ T cells did not have any effect (Figure 9).

Depletion of γδ T cells in long-term challenged mice did not have a significant effect on the cytokines IL-4, IL-5, and IFN-γ in BALF (data not shown). However, IL-10 and IL-12, which were increased in these mice, reverted to background levels after γδ T cell depletion (Figure 10).

Finally, despite the reappearance of airway hyperreactivity to inhaled MCh, depletion of γδ T cells, including general depletion or depletion of $V\gamma4^+$ cells specifically, did not significantly affect eosinophilic airway inflammation in long-term challenged mice; it remained at low levels although increased over background levels (Figure 11).

DISCUSSION

Long-term allergen challenge can reduce allergic hyperresponsiveness in different species, including humans (7–9), but the underlying mechanisms are not well defined. In the current study,

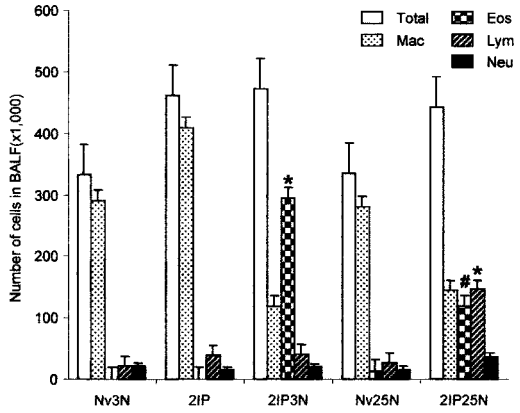

Figure 4. Bronchoalveolar lavage fluid (BALF) cells (short versus long): Cell composition in BALF. Data represent means ± SEM for the groups shown in Figure 2. *Significant difference ($p < 0.05$) between short-term challenged mice (2IP3N) and long-term challenged (2IP25N) and controls; #significant difference ($p < 0.05$) between sensitized and long-term challenged mice (2IP25N) and short-term challenged mice (2IP3N) and controls. Eos = eosinophils; Lym = lymphocytes; Mac = macrophages; Neu = neutrophils.

we established a model in which mice, hyperreactive to OVA as a result of prior sensitization and airway challenge, were restored to normal responsiveness after repeated challenge with aerosolized OVA. This model is characterized not only by a full reversal of airway hyperreactivity to inhaled MCh, but also by a large reduction in eosinophilic airway inflammation, when compared with controls.

The return to normal airway responsiveness was associated with a change in cytokine profile in BALF from a Th2- to a Th1-biased pattern, suggestive of active immunoregulation as opposed to an exhaustion of the host response. Consistently, pulmonary lymphocytes transferred from the long-term challenged mice suppressed airway responses in hyperreactive recipients, ruling out passive mechanisms of nonresponsiveness as, for example, antigen-specific tolerance and depletion/anergy

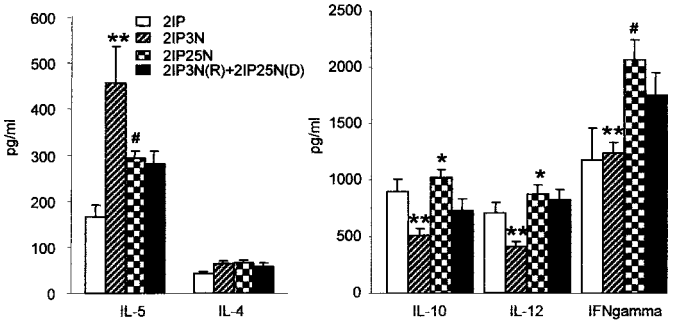

Figure 5. Cytokines in BALF (transfer). Levels of IL-4, IL-5, IL-10, IL-12, and IFN-γ in BALF in the groups shown in Fig. 2 as well as after adoptive transfer (R = recipients; D = donor). Data represent means ± SEM (n = 8–14 mice in each group). *Significant difference ($p < 0.05$) between sensitized and long-term challenged mice (2IP25N) and short-term challenged mice (2IP3N); #significant difference ($p < 0.05$) between sensitized and long-term challenged mice (2IP25N) and controls and between short-term challenged mice (2IP3N) and controls; **significant difference ($p < 0.05$) between nonrecipients (2IP3N) and recipients [2IP3N(R)+2IP25N(D)] and long-term challenged mice (2IP25N).

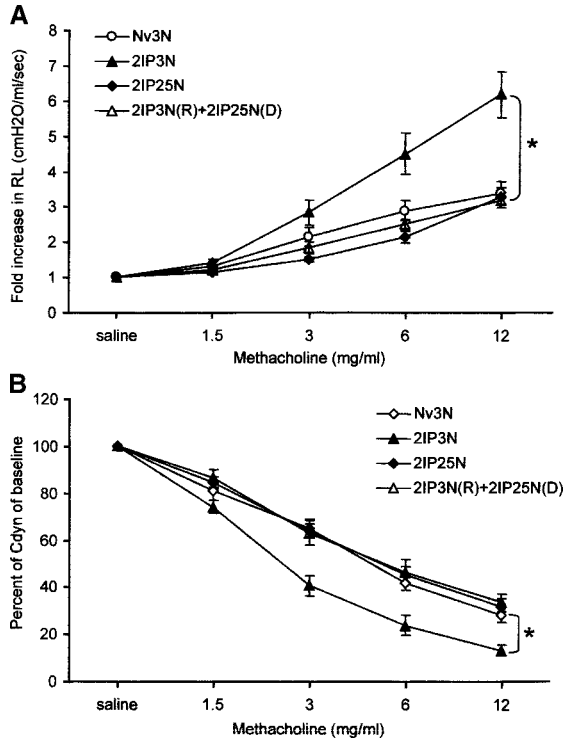

Figure 6. AHR (transfer). Increases in R_L (*A*) and decrease in C_{Ldyn} (*B*) after adoptive cell transfer. Data represent means ± SEM (n = 8–14 mice in each group). Baseline values (R_L and C_{Ldyn}) were not significantly altered between groups. *Significant difference ($p < 0.05$) between nonrecipients (2IP3N) and recipients [2IP3N (R)+2IP25N (D)]. D = donor; Nv = nonsensitized; R = recipient.

of OVA-reactive αβ T cells (28–30). Although these adoptive transfer experiments support an active suppression, in and of themselves, they do not directly implicate γδ T cells in the process. However, the transferred cells had no significant effect on

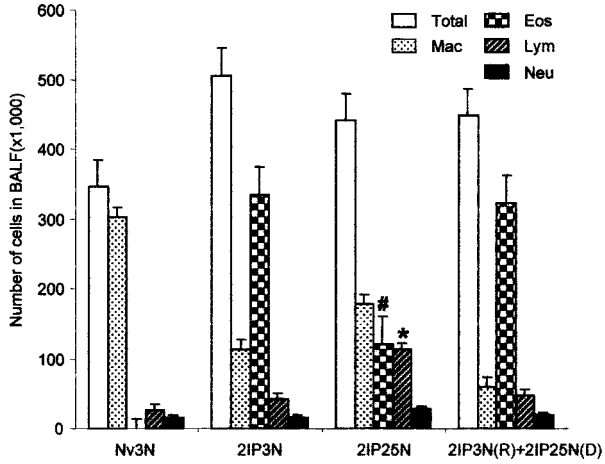

Figure 7. BALF cells (transfer): Cell composition in BALF. Data represent means ± SEM. The groups are the same as in Figure 6. #Significant difference ($p < 0.05$) between long-term challenged mice and short-term challenged mice and controls; *significant difference ($p < 0.05$) between long-term challenged mice and short-term challenged mice and controls.

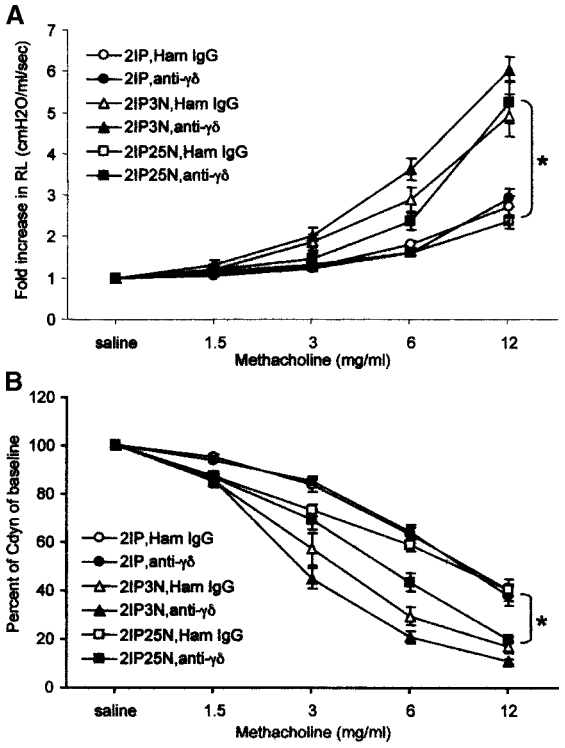

Figure 8. AHR (pan-depletion): Increase in RL (*A*) and decrease in CLdyn (*B*) after γδ T cell depletion. Data represent means ± SEM (n = 8–12 mice in each group). Baseline values for RL and CLdyn were not significantly different between groups. *Significant difference ($p < 0.05$) between long-term challenged mice with and without depletion of γδ T cells.

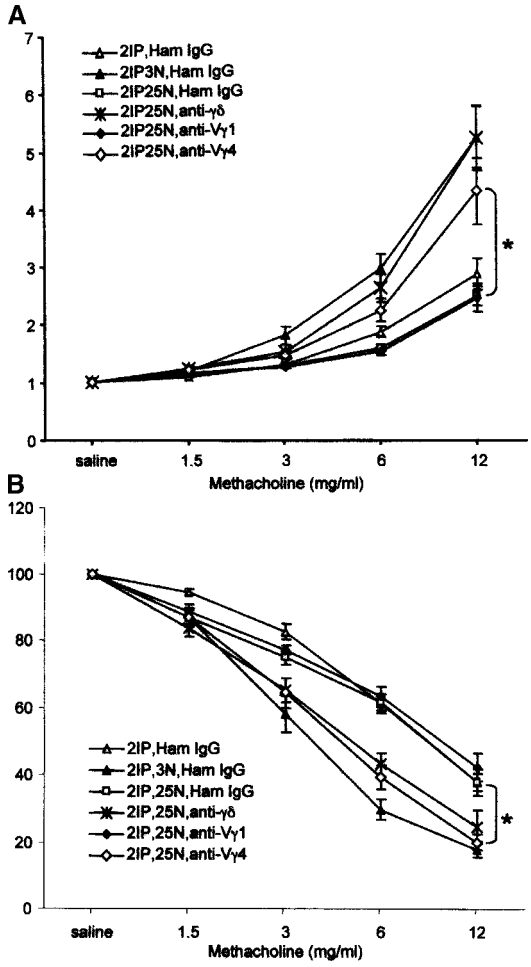

Figure 9. AHR (subset depletion): Increase in RL (*A*) and CLdyn (*B*) after γδ T cell depletion. Data represent means ± SEM (n = 8–14 mice in each group). *Significant difference ($p < 0.05$) between long-term challenged mice and after depletion of $V\gamma4^+$ cells or γδ T cells.

eosinophilic airway inflammation in the allergic/hyperreactive recipients, suggesting that reduction of airway inflammation after long-term allergen challenge is governed by entirely different regulatory mechanisms.

In this model, we further found that γδ T cells are required for the inhibitory effect of long-term allergen exposure on AHR to MCh, but not for the reduction of eosinophilic airway inflammation. In fact, γδ T cells appeared solely responsible for the regulatory effect on AHR under these conditions, because their depletion fully restored hyperreactivity. On the other hand, there was no indication that γδ T cells played any part in the regulatory effects of long-term allergen exposure on eosinophilia in BALF. This comes as a surprise given that in infectious inflammation, a regulatory role for γδ T cells has been documented repeatedly (24, 31). Perhaps, inflammation must be of a certain kind or mediated by a specific group of mediators, to be regulated by γδ T cells. In either case, these findings may help to explain conflicting observations with regard to the involvement of γδ T cells in different rodent models of asthma (12, 32), as it appears that not all parameters examined are likely to reveal the influence of these cells. Finally, there was no indication that γδ T cells had an overall influence on the reversed Th cytokine shift (involving IL-4, IL-5, and IFN-γ; Th2 to Th1) observed in long-term challenged mice. Direct regulatory effects on OVA-specific αβ T cells thus do not seem to play a role in this model of long-term allergen exposure. We previously showed that the regulatory effects of γ/δ T cells were exhibited in an α/β T cell-independent manner (12). On the other hand, γδ T cells were obviously required for the development of increased IL-10 and IL-12 levels after the long-term allergen challenge, suggestive of potential regulatory interactions with the innate system, including perhaps macrophages and dendritic cells, known to produce these cytokines in quantity (33–35). Cumulatively, these findings may reflect a role for γδ T cells in an innate regulatory circuit capable of protecting normal airway responsiveness in the presence or absence of airway inflammation, as we have argued previously (12, 13), studying different models of AHR.

We have previously reported that AHR is increased in OVA-sensitized and challenged mice when γδ T cells are absent (12). Moreover, even in mice deficient in αβ T cells and thus incapable of developing an OVA-specific allergic response, we still found increased airway responses after OVA challenge when γδ T cells were absent (12). In both models, including adoptive transfer, $V\gamma4^+$ γδ T cells appear to be responsible for the protective effect (27, 36), and in the current study the same subset appears to mediate the suppressive effect of long-term allergen challenge on AHR. We have shown that the $V\gamma4^+$ regulatory subset also expresses CD8β and produces IFN-γ (27). The negative regulatory function of these cells is independent of αβ T cells, was abrogated in MHC class I-deficient mice, and requires IFN-γ (27). The new data imply that these same cells are engaged in the active reversal of AHR after long-term allergen challenge, even in mice with established allergic responses.

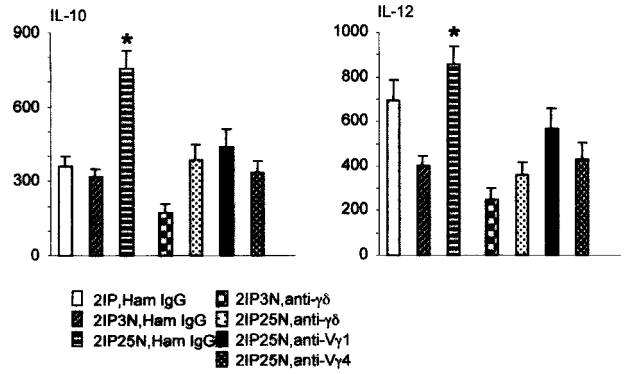

Figure 10. ILs in BALF (depletion): Levels of IL-10 and IL-12 in BALF. Data represent means ± SEM (n = 8–10 mice in each group). Depletion of γδ T cells decreased the levels of IL-10 and IL-12 in BALF in long-term challenged mice. *Represents significant difference ($p < 0.05$) between long-term challenged mice (2IP25N, control) and γδ T cells depletion.

It is a distinctive feature of γδ T cells that TCR-defined subsets are segregated both in terms of development, tissue distribution, and even function (37). In mice, Vγ expression serves as a reliable marker of functionally segregated subsets. The lungs of normal adult mice are colonized by approximately 5×10^4 γδ T cells. Roughly one-third of these are $V\gamma4^+$, and only a fraction of the $V\gamma4^+$ pulmonary subset expresses CD8. In an experimental model involving only three airway challenges without prior sensitization, these cells did not increase significantly in the lung. Nevertheless, their local depletion with aerosolized antibodies specific for Vγ4 resulted in AHR (27). Therefore, the regulatory subset in the lung appears to be small. This observation is reminiscent of a study published by McMenamin and coworkers (38), who described the effects of repeated airway exposure to OVA in C57BL/6 mice. Small numbers of splenic γδ T cells, isolated from these mice, suppressed primary IgE production in adoptive cell transfer recipients, and a similar finding was later reported with rats (39). It seems likely that the mechanism of active suppression they described, and that of active AHR suppression described here, involve related populations of regulatory γδ T cells. The new data are consistent with this interpretation. We suspect that in OVA-specific and challenged, hyperreactive mice, the small endogenous population of regulatory γ/δ T cells is not adequate to prevent AHR, although it may reduce it. Increasing the size of the regulatory population after repeated airway challenge or by cell transfer could alter this balance, restoring normal airway responsiveness.

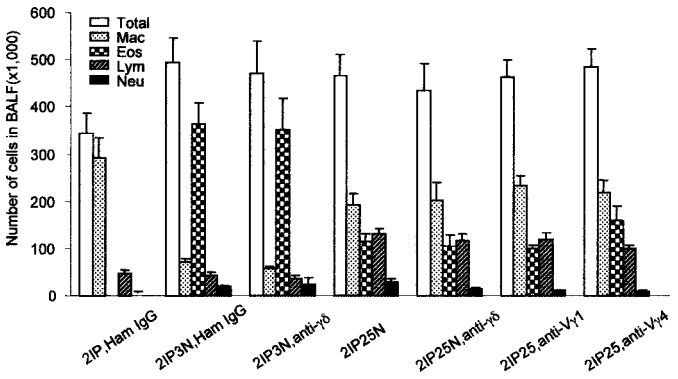

Figure 11. BAL cells (depletion). Cell composition in BALF. Data represent means ± SEM. The groups are the same as the Figure 10.

Our data suggest that the regulatory effects of γδ T cells are restricted to certain parameters of the allergic response. Given the dissociation between the effects of $V\gamma4^+$ T cells on airway function and airway inflammation, it is possible that these T cells impact airway smooth muscle function directly (e.g., through adhesion molecule interactions) or via cytokines or other intermediates. Airway smooth muscle cells are themselves capable of secreting a number of cytokines in an autocrine fashion, and when activated lead to increased bronchoconstrictor responsiveness (40–43). We have shown that adoptive transfer of $STAT6^{+/+}$ T cells from sensitized and challenged mice into $STAT6^{-/-}$ mice exposed to airway challenge alone can develop AHR in the absence of airway inflammation, lending support to a more direct influence of activated T cells on airway smooth muscle function (44). A number of cytokines including IL-1β, IL-10, IL-5, and IL-13 have been implicated in airway smooth muscle contraction (40–43). At present, it is unclear whether changes in any of the cytokines measured as a consequence of γδ T cell depletion, IL-10, IL-12, or IFN-γ, are mediators of this γδ-dependent regulation of airway responsiveness. Administration of any of these cytokines has been associated with a reduction in AHR, accompanied by a reduction in airway inflammation. The reduction in goblet cell hyperplasia with long-term challenge is indicative of a reduction in IL-13, a central cytokine in this response (45, 46).

The regulation of airway responsiveness by activated γδ T cells, independent of αβ T cells, has now been described in several different models and in at least two strains of mice, BALB/c and C57BL/6 (12). These regulatory effects do not involve changes in the inflammatory response but appear to directly impact airway function. A major regulator of γδ cell activation is tumor necrosis factor-α (TNF-α). TNF-α rapidly increases in sensitized mice after allergen challenge. In overexpressing, TNF-α transgenic mice, AHR failed to develop (47). Depletion of γδ T cells in these mice restored AHR (47). This link between TNF-α and γδ regulatory function was extended by examining TNF-α receptor–deficient mice. TNFR1 (p55)-deficient mice (unopposed p75 activation) failed to develop AHR, in contrast to p75-deficient mice. Depletion of γδ T cells in the p55-deficient mice restored development of AHR; the p75-deficient mice were unaffected by this depletion (48). In each of these cases, restoration of AHR was independent of effects on the lung eosinophil inflammatory response. By linking these results with those in the present study, we speculate that repeated challenge of sensitized mice leads to expansion and activation of γδ T cells, and of $V\gamma4^+$ cells in particular, through interactions of TNF-α and the p75 receptor, resulting in the downregulation of AHR.

Potentially, a second link in the regulatory activation of γδ T cells is through the induction of T-bet (T-box expressed in T cells), a newly identified Th1-specific transcription factor (49). T-bet, absent in naive γδ T cells, is induced on activation, resulting in IFN-γ production. In γδ T cells, T-bet–dependent IFN-γ production does not appear to be counterbalanced by GATA-3, unlike in αβ T cells (50, 51). TNF-α, released in sensitized/challenged mice, through p75 could induce T-bet, resulting in increased IFN-γ production.

In summary, repeated exposure of sensitized mice to inhaled allergen results in marked changes in cytokine, inflammatory cell, and airway responses. A shift toward a Th1-biased response, reduced lung eosinophilia, and normalization of airway responsiveness is demonstrated. Adoptive transfer of lung T cells from these mice results in a similarly altered phenotype in sensitized

and challenged (short-term) recipient mice. Repetitive airway challenge leads to an increase in lung γδ T cells and these cells, in particular $V\gamma4^+$ T cells, appear responsible for the regulation of airway responsiveness with little or no effect on airway inflammation. Airway inflammation in this model may be entirely under the control of αβ T cells, and because we have shown that the effects of γδ T cells, including $V\gamma4^+$ cells, are independent of αβ T cells (12, 27), this may explain the dissociation of the regulatory effects. Moreover, it suggests that targeting or manipulating one without the other can have important consequences on lung function.

Conflict of Interest Statement: Z-H.C. has no declared conflict of interest; A.J. has no declared conflict of interest; M.K.A. has no declared conflict of interest; Y-S.H. has no declared conflict of interest; W.K.B. has no declared conflict of interest; E.W.G. has no declared conflict of interest.

Acknowledgment: The authors thank Diana Nabighian for her assistance.

References

1. Brusselle G, Kips J, Joos G, Bluethmann H, Pauwels R. Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *Am J Respir Cell Mol Biol* 1995;12: 254–259.
2. Takeda K, Hamelmann E, Joetham A, Shultz LD, Larsen GL, Irvin CG, Gelfand EW. Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. *J Exp Med* 1997;186:449–454.
3. Inman MD, Ellis R, Wattie J, Denburg JA, O'Byrne PM. Allergen-induced increase in airway responsiveness, airway eosinophilia, and bone-marrow eosinophil progenitors in mice. *Am J Respir Cell Mol Biol* 1999;21:473–479.
4. Lee NA, Gelfand EW, Lee JJ. Pulmonary T cells and eosinophils: coconspirators or independent triggers of allergic respiratory pathology. *J Allergy Clin Immunol* 2001;107:945–957.
5. Schuh JM, Blease K, Kunkel SL, Hogaboam CM. Eotaxin/CCL11 is involved in acute, but not chronic, allergic airway responses to *Aspergillus fumigatus*. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L198–L204.
6. Blease K, Jakubzick C, Westwick J, Lukacs N, Kunkel SL, Hogaboam CM. Therapeutic effect of IL-13 immunoneutralization during chronic experimental fungal asthma. *J Immunol* 2001;166:5219–5224.
7. Sakai K, Yokoyama A, Kohno N, Hamada H, Hiwada K. Prolonged antigen exposure ameliorates airway inflammation but not remodeling in a mouse model of bronchial asthma. *Int Arch Allergy Immunol* 2001;126:126–134.
8. Jungsuwadee P, Dekan G, Stingl G, Epstein MM. Recurrent aerosol antigen exposure induces distinct patterns of experimental allergic asthma in mice. *Clin Immunol* 2002;102:145–153.
9. Palmqvist M, Cui Z-H, Sjostrand M, Linden A, Lotvall J. Reduced late asthmatic response by repeated low-dose allergen exposure. *Eur Respir J* 2001;17:872–880.
10. Knott PG, Gater PR, Bertrand CP. Airway inflammation driven by antigen-specific resident lung $CD4^+$ T cells in αβ-T cell receptor transgenic mice. *Am J Respir Crit Care Med* 2000;161:1340–1348.
11. Knott PG, Gater PR, Dunford PJ, Fuentes ME, Bertrand CP. Rapid upregulation of CXC chemokines in the airways after Ag-specific $CD4^+$ T cell activation. *J Immunol* 2001;166:1233–1240.
12. Lahn M, Kanehiro A, Takeda K, Joetham A, Schwarze J, Kohler G, O'Brien RL, Gelfand EW, Born WK. Negative regulation of airway responsiveness that is dependent on γδ T cells and independent of αβ T cells. *Nat Med* 1999;5:1150–1156.
13. Born WK, Lahn M, Takeda K, Kanehiro A, O'Brien RL, Gelfand EW. Role of γδ T cells in protecting normal airway function. *Respir Res* 2000;1:151–158.
14. Pawankar R. γδ T cells in allergic airway diseases. *Clin Exp Allergy* 2000; 30:318–323.
15. Wills-Karp M. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu Rev Immunol* 1999;17:255–281.
16. Taube C, Duez C, Cui Z-H, Takeda K, Rha Y-H, Park JW, Balhorn A, Donaldson DD, Dakhama A, Gelfand EW. The role of IL-13 in established allergic airway disease. *J Immunol* 2003;169:6482–6489.
17. Kuribayashi K, Kodama T, Okamura H, Sugita M, Matsuyama T. Effects of post-inhalation treatment with interleukin-12 on airway hyper-reactivity, eosinophilia and interleukin-18 receptor expression in a mouse model of asthma. *Clin Exp Allergy* 2002;32:641–649.
18. Tournoy KG, Kips JC, Pauwels RA. Counterbalancing of T_H2-driven allergic airway inflammation by IL-12 does not require IL-10. *J Allergy Clin Immunol* 2001;107:483–491.
19. Lack G, Bradley KL, Hamelmann E, Renz H, Loader J, Leung DYM, Larsen GL, Gelfand EW. Nebulized IFN-γ inhibits the development of secondary allergic responses in mice. *J Immunol* 1996;157:1432–1439.
20. Suzuki M, Maghni K, Molet S, Shimbara A, Hamid QA, Martin JG. IFN-γ secretion by $CD8^+$ T cells inhibits allergen-induced airway eosinophilia but not late airway responses. *J Allergy Clin Immunol* 2002;109:803–809.
21. Cohn L, Herrick C, Niu N, Homer R, Bottomly K. IL-4 promotes airway eosinophilia by suppressing IFN-γ production: defining a novel role for IFN-γ in the regulation of allergic airway inflammation. *J Immunol* 2001;166:2760–2767.
22. Martin TR, Gerard NP, Galli SJ, Drazen JM. Pulmonary responses to bronchoconstrictor agonists in the mouse. *J Appl Physiol* 1988;64: 2318–2323.
23. Oshiba A, Hamelmann E, Takeda K, Bradley KL, Loader JE, Larsen GL, Gelfand EW. Passive transfer of immediate hypersensitivity and airway hyperresponsiveness by allergen-specific immunoglobulin (Ig) E and IgG1 in mice. *J Clin Invest* 1996;97:1398–1408.
24. O'Brien RL, Yin X, Huber SA, Ikuta K, Born WK. Depletion of a γδ T cell subset can increase host resistance to a bacterial infection. *J Immunol* 2000;165:6472–6479.
25. Dent AL, Matis LA, Hooshmand F, Widacki SM, Bluestone JA, Hedrick SM. Self-reactive γδ T cells are eliminated in the thymus. *Nature* 1990;343:714–719.
26. Pereira P, Gerber D, Huang SY, Tonegawa S. Ontogenic development and tissue distribution of Vγ1-expressing γ/δ T lymphocytes in normal mice. *J Exp Med* 1995;182:1921–1930.
27. Lahn M, Kanehiro A, Takeda K, Terry J, Hahn YS, Aydintug MK, Konowal A, Ikuta K, O'Brien RL, Gelfand EW, *et al.* MHC class I-dependent $V\gamma4^+$ pulmonary T cells regulate αβ T cell-independent airway responsiveness. *Proc Natl Acad Sci USA* 2002;99:8850–8855.
28. Seymour BW, Gershwin LJ, Coffman RL. Aerosol-induced immunoglobulin (Ig)-E unresponsiveness to ovalbumin does not require $CD8^+$ or T cell receptor (TCR)-γ/δ^+ T cells or interferon (IFN)-γ in a murine model of allergen sensitization. *J Exp Med* 1998;187:721–731.
29. Tsitoura DC, DeKruyff RH, Lamb JR, Umetsu DT. Intranasal exposure to protein antigen induces immunological tolerance mediated by functionally disabled $CD4^+$ T cells. *J Immunol* 1999;163:2592–2600.
30. Tsitoura DC, Blumenthal RL, Berry G, Dekruyff RH, Umetsu DT. Mechanisms preventing allergen-induced airways hyperreactivity: role of tolerance and immune deviation. *J Allergy Clin Immunol* 2000;106: 239–246.
31. Roark CE, Vollmer MK, Campbell PA, Born WK, O'Brien RL. Response of a $\gamma\delta^+$ T cell receptor invariant subset during bacterial infection. *J Immunol* 1996;156:2214–2220.
32. Zuany-Amorim C, Ruffie C, Haile S, Vargaftig BB, Pereira P, Pretolani M. Requirement for γδ T cells in allergic airway inflammation. *Science* 1998;280:1265–1267.
33. Sirois J, Bissonnette EY. Alveolar macrophages of allergic resistant and susceptible strains of rats show distinct cytokine profiles. *Clin Exp Immunol* 2001;126:9–15.
34. Zeibecoglou K, Ying S, Meng Q, Poulter LW, Robinson DS, Kay AB. Macrophage subpopulations and macrophage-derived cytokines in sputum of atopic and nonatopic asthmatic subjects and atopic and normal control subjects. *J Allergy Clin Immunol* 2000;106:697–704.
35. Magnan A, van Pee D, Bongrand P, Vervloet D. Alveolar macrophage interleukin (IL)-10 and IL-12 production in atopic asthma. *Allergy* 1998;53:1092–1095.
36. Hahn Y-S, Taube C, Jin N, Takeda K, Park JW, Wands JM, Aydintug MK, Roark CL, Lahn M, O'Brien RL, *et al.* $V\gamma4^+$ T cells regulate hyperreactivity to methacholine in ovalbumin-sensitized and challenge mice. *J Immunol* 2003;171:3170–3178.
37. O'Brien RL, Lahn M, Born WK, Huber SA. T cell receptor and function cosegregate in γ-δ T cell subsets. *Chem Immunol* 2001;79:1–28.
38. McMenamin C, Pimm C, McKersey M, Holt PG. Regulation of IgE responses to inhaled antigen in mice by antigen-specific γδ T cells. *Science* 1994;265:1869–1871.
39. McManamin C, McKersey M, Kuhnlein P, Hunig T, Holt PG. γ/δ T cells downregulate primary IgE responses in rats to inhaled soluble protein antigens. *J Immunol* 1995;154:4390–4394.
40. Reynolds AM, Holmes MD, Scicchitano R. Cytokines enhance airway

smooth muscle contractility in response to acetylcholine and neurokinin. *Respirology* 2000;5:153–160.
41. Grunstein MM, Hakonarson H, Leiter J, Chen M, Whelan R, Grunstein JS, Chuang S. Autocrine signaling by IL-10 mediates altered responsiveness of atopic sensitized airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2001;281:L1130–L1137.
42. Hakonarson H, Maskeri N, Carter C, Grunstein MM. Regulation of T_H1- and T_H2-type cytokine expression and action in atopic asthmatic sensitized airway smooth muscle. *J Clin Invest* 1999;103:1077–1087.
43. Grunstein MM, Hakonarson H, Leiter J, Chen M, Whelan R, Grunstein JS, Chuang S. IL-13-dependent autocrine signaling mediates altered responsiveness of IgE-sensitized airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L520–L528.
44. Tomkinson A, Duez C, Lahn M, Gelfand EW. Adoptive transfer of T cells induces airway hyperresponsiveness independently of airway eosinophilia but in a signal transducer and activator of transcription 6-dependent manner. *J Allergy Clin Immunol* 2002;109:810–816.
45. Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, Elias JA, Sheppard D, Erle DJ. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* 2002;8:885–889.
46. Lee CG, Homer RJ, Cohn L, Link H, Jung S, Craft JE, Graham BS, Johnson TR, Elias JA. Transgenic overexpression of IL-10 in the lung causes mucus metaplasia, tissue inflammation and airways remodeling via IL-13-dependent and -independent pathways. *J Biol Chem* 2002; 277:35466–35474.
47. Kanehiro A, Lahn M, Makela MJ, Dakhama A, Fujita M, Joetham A, Mason RJ, Born WK, Gelfand EW. Tumor necrosis factor-α negatively regulates airway hyperresponsiveness through γ-δ T cells. *Am J Respir Crit Care Med* 2001;164:2229.
48. Kanehiro A, Lahn M, Makela MJ, Dakhama A, Joetham A, Rha Y-H, Born WK, Gelfand EW. Requirement for the p75 TNF-α receptor (TNFR2) in the regulation of airway hyperresponsiveness by γδ T cells. *Am J Respir Crit Care Med* 2001;164:2229–2238.
49. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000;100:655–669.
50. Lighvani AA, Frucht DM, Jankovic D, Yamane H, Aliberti J, Hissong BD, Nguyen BV, Gadina M, Sher A, Paul WE, *et al.* T-bet is rapidly induced by interferon-γ in lymphoid and myeloid cells. *Proc Natl Acad Sci USA* 2001;98:15137–15142.
51. Yin Z, Chen C, Szabo SJ, Glimcher LH, Ray A, Craft J. T-Bet expression and failure of GATA-3 cross-regulation lead to default production of IFN-γ by γδ T cells. *J Immunol* 2002;168:1566–1571.